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(19) (CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Angiotensin II Type-1 Receptor and Its Production

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ABSTRACT

The present invention relates to (1) a human angiotensin II type 1 receptor protein, a recombinant DNA containing a gene which codes for said protein, a transformant carrying said DNA, a production of said protein and anti-angiotensin II substance screening methods using said transformant containing said protein.

Angiotensin II type 1 Receptor and Its Production

Field of the invention

5 The present invention relates to a new human angiotensin II type 1 receptor, a recombinant DNA which codes therefor, a transformant carrying said recombinant DNA, a method of producing said receptor, and a use therefor. More specifically, the present invention provides a method of accurately assaying the bioactivities of angiotensin II antagonists and
10 agonists, in a pure system containing substantially no other receptors, by expressing a human angiotensin II type 1 receptor gene in an animal cell using recombinant DNA technology.

Background of the invention

15 The renin-angiotensin (RA) system, essential to the regulation of blood pressure and aqueous electrolytes in vivo, plays a key role in various hypertensive diseases, congestive heart failure and edematous diseases.

 Renin, produced mainly in the renal juxtaglomerular apparatus, acts on angiotensinogen, present in the blood, kidney and other organs, to produce
20 angiotensin (Ang) I. Ang I possesses almost no bioactivity and, upon action of angiotensin-converting enzyme (ACE), is converted to a bioactive form known as Ang II. Ang II is thought to make a most significant contribution to the bioactivity of the RA system, though Ang III is also produced by the same system. The amino acid number of Ang III is lower by 1 than that of Ang II,
25 and its bioactivity is similar to that of Ang II.

 The most important action of Ang II is its cardiovascular action, its peripheral vasoconstrictive action being very potent and playing a major role in the maintenance of blood pressure. In addition to this action, Ang II has proven active on the adrenal zona glomerulosa to induce aldosterone
30 production and on the adrenal medulla and sympathetic nerve ends to promote catecholamine secretion, vasopressin secretion and prostaglandin E2 and I2 production, and is involved in the glomerular filtering function and the renal uriniferous tubular sodium reabsorption mechanism. Since renin and Ang II are also produced in the brain, heart, vascular wall, adrenal and other
35 non-kidney organs, the local action thereof as produced in these ectopic RA systems is drawing attention, as are the above physiological actions.

Since the RA system owes its bioactivity mainly to Ang II, as stated above, it has been believed that the above-mentioned diseases can be prevented or mitigated by suppressing Ang II production and hence the RA system. To inhibit the renin production, which is the starting material of the RA system, β -blockers have long been used. However, since β -blockers possess a broad range of action points, focus has recently been on the development of renin inhibitors, which are unsatisfactory in absorption via the digestive tract; no drug permitting practical application has been developed. Currently widely used RA system inhibitors are ACE inhibitors, exemplified by captopril, enalapril, delapril and alacepril. Although these drugs are already in practical application for their excellent effect, they have side effects, such as dry cough and diuresis, as a result of increase in bradykinin and prostaglandin levels, because they also suppress kininase II. Therefore the development of an Ang II receptor antagonist has been desired as a drug suppressing only Ang II bioactivity.

Orally administrable non-peptide Ang II receptor antagonists have long been studied. For example, imidazole acetate series compounds have been studied for diuretic and hypotensive action since around 1976, and; CV-2961 and other compounds have been found to possess Ang II receptor antagonist activity [Y. Furukawa et al., US Patent No. 4,340,598 (1982); Y. Furukawa et al., US Patent No. 4,355,040 (1982)], as the result. Since then, there have been improvements in these compounds, resulting in the development of DuP 753 [A.T. Chiu et al., Journal of Pharmacology and Experimental Therapeutics, 252, 711 (1990)]. DuP 753 proved to active specifically on Ang II receptors in the vascular wall, adrenal cortex and other organs to suppress Ang II action only. It was also shown to have no effect on reactions of KCl, norepinephrine, isoproterenol, vasopressin, bradykinin, acetylcholine or 5-HT, or on ACE action, even at suppressive doses for a high concentration (10 μ M) of Ang II [P.C. Wong et al., J. Pharmacol. Exp. Ther., 252, 719 (1990)]. According to the recognition of such pharmacological utility of non-peptide antagonists for Ang II receptors, the studies of Ang II receptors has been increased. It is conjectured that there are at least two kinds of Ang II receptor, according to reactivity to antagonists: type 1 receptors, to which DuP 753 is antagonistic, and type 2 receptors, to which PD123177 is

antagonistic [P.B.M.W.M. Timmermans et al., Trends in Pharmaceutical Science (TiPS), 12, 55 (1991)], of which type 1 receptors are thought to play a key role in known Ang II-dependent diseases.

5 Thus development of Ang II type 1 receptor antagonists have been brisk. To accurately assess the antagonist activity of a drug, it is necessary to use cells or cell membrane fraction having Ang II type 1 receptors only. However, the receptors used in these studies are based on membrane fractions derived from laboratory animals (e.g., bovines, rats), which contain various
10 receptors other than the desired Ang II receptor. For this reason, there is demand for preparing cells which specifically express human Ang II type 1 receptors and use such cells or cell membrane fractions to accurately determine the bioactivity of the antagonist in a pure system.

15 Summary of the invention

 The present inventors have succeeded in cloning a gene from a cDNA library derived from the human placenta which shares high homology with known human, bovine and rat Ang II type 1 receptor genes but which has a new nucleotide sequence. The inventors expressed this clone in an animal
20 cell and found that the clone can serve as an Ang II type 1 receptor. Assuming that the receptor thus obtained is an Ang II type 1 receptor of human origin, the inventors made further investigations based on the above findings and developed the present invention.

25 Accordingly, the present invention comprises (1) synthesizing a primer for amplifying a well-conserved region, based on the reported sequences for the Ang II type 1 receptor derived from bovine adrenal zona glomerulosa cells [K. Sasaki et al., Nature, 351, 230 (1991)] and for the Ang II type 1 receptor derived from rat aortic smooth muscle cells [T.J. Murphy et al., Nature, 351,
30 233 (1991)], (2) amplifying the well-conserved region from a cDNA library derived from the human placenta by PCR using said primer, (3) subcloning of the amplified DNA into a vector, (4) identifying the region which codes for the human Ang II type 1 receptor from the subcloned DNA, (5) obtaining a novel Ang II type 1 receptor gene of human origin by plaque hybridization using
35 said coding sequence as a probe, (6) preparing a recombinant DNA for expressing the gene of (5) above in a host cell, (7) preparing a transformant

carrying the recombinant DNA of (6) above, (8) obtaining the Ang II type 1 receptor of human origin by culturing the transformant of (7) above, (9) determining the bioactivity of a receptor antagonist using all or part (e.g., cell membrane) of the transformant of (7) above, and (10) determining the activity of the Ang II type 1 receptor of human origin using the transformant of (7) above.

Brief Description of the Drawings

Fig. 1 shows the nucleotide sequence of the Ang II type 1 receptor gene, derived from human placenta, contained in the plasmid pHARp116 (see Example 3).

Fig. 2 shows the amino acid sequence deduced from the Ang II type 1 receptor gene, derived from human placenta, contained in the plasmid pHARp116 (see Example 3).

Fig. 3 shows changes in intracellular calcium ion concentration in response to stimulation with Ang II in full-length heart-derived Ang II type 1 receptor expressing cells (see Example 11).

Fig. 4 shows changes in intracellular calcium ion concentration in response to stimulation with Ang II in full-length placenta-derived Ang II type 1 receptor expressing cells (see Example 11).

Fig. 5 shows dose-response curve of intracellular calcium ion concentration to stimulation with Ang II in full-length Ang II type 1 receptor expressing cells.

Fig. 6 shows changes in intracellular inositol phosphate concentration in response to stimulation with Ang II in full-length Ang II type 1 in receptor expressing cells.

Fig. 7 shows dose-response curve of intracellular inositol-phosphate concentration to stimulation with Ang II in full-length Ang II type 1 receptor expressing cells.

Fig. 8 shows changes in intracellular cyclic AMP concentration in response to stimulation with Ang II in full-length Ang II type-1 receptor expressing cells.

Detail description of the invention

The present invention provides:

(1) a new polypeptide in a new human Ang II type 1 receptor, i.e., polypeptide (I) comprising the amino acid sequence represented by the following formula I:

5 Arg-Asn-Ser-Thr-Leu-Pro-Ile-Gly-Leu-Gly-Leu-Thr-Lys-Asn-Ile-Leu-Gly-Ser-
Cys-Phe-Pro-Phe-Leu-Ile-Ile-Leu-Thr-Ser-Tyr-Thr-Leu-Ile-Trp-Lys-Ala-Leu-
Lys-Lys-Ala-Tyr-Glu-Ile-Gln-Lys-Asn-Asn-Pro-Arg-Asn-Asp-Asp-Ile-Phe-Arg-
Ile-Ile-Met-Ala-Ile-Val-Leu-Phe-Phe-Phe-Phe-Ser-Trp-Ile-Pro-His-Gln-Ile-
10 Phe-Thr-Phe-Leu-Asp-Val-Leu-Ile-Gln-Gln-Gly-Ile-Ile-Arg-Asp-Cys-Arg-Ile-
Ala-Asp-Ile-Val-Asp-Thr-Ala-Met-Pro-Ile-Thr-Ile-Trp-Ile-Ala-Tyr-Phe-Asn-
Asn-Cys-Leu-Asn-Pro-Leu-Phe-Tyr-Gly-Phe-Leu-Gly-Lys-Lys-Phe-Lys-Lys-Asp-
15 Ile

(SEQ ID No. 1),

(2) a recombinant DNA (A) which encodes polypeptide (I),

(3) a transformant (B) carrying a vector comprising said recombinant
20 DNA (A),

(4) a method of producing polypeptide (I) wherein transformant (B) is cultured in a medium to produce and accumulate polypeptide (I), which is then harvested,

(5) a method of screening an anti-angiotensin II substance, which
25 comprises in the presence of angiotensin II and the polypeptide (I), or searching a substance capable of inhibiting angiotensin II from binding to said polypeptide.

(6) A method of screening an angiotensin II type 1 receptor ligand which comprises searching a substance capable of binding the polypeptide (I).

(7) A method of screening an anti-angiotensin II substance, which
30 comprises in the presence of the transformant (B) which contains the polypeptide (I) and angiotensin II, or searching a substance capable of suppressing change of a concentration of an angiotensin II responsive substance intracellularly in response of angiotensin II existing in said
35 transformant.

(8) A method of screening an angiotensin II type 1 receptor agonist which comprises searching a substance capable of changing a concentration of an angiotensin II responsive substance intracellularly in the transformant (B) which consists the polypeptide (I).

5

Any polypeptide can serve as polypeptide (I), as long as it is capable of receiving human Ang II, i.e. specifically binds to ligands capable of binding to human Ang II type 1 receptors, such as human Ang II antagonists and agonists for human Ang II type 1 receptor, and inducing activation of intracellular substances (e.g., Ca ions) or behavior change by the resulting structural change, and contains the amino acid sequence represented by the above formula (I) (SEQ ID No. 1). Such polypeptides include (1) human Ang II type 1 receptor comprising the amino acid sequence shown in Fig. 2 (SEQ ID No. 6), and (2) human Ang II type 1 receptor muteins such as proteins resulting from deletion or replacement of one or more of the amino acids at the 1-186 and 314-359 positions in the amino acid sequence shown in Fig. 2, and proteins resulting from addition of one or more amino acids to the N terminal or C terminal of the amino acid sequence shown in Fig. 2, with preference given to human Ang II type 1 receptor represented by the amino acid sequence shown in Fig. 2 (SEQ ID No. 6).

Any DNA can serve as the recombinant DNA (A), as long as it contains a nucleotide sequence which codes for the amino acid sequence represented by formula I, whether part or all of the sequence is derived from a natural source or chemical synthesis, or combination of them.

25

Any nucleotide sequence can serve to code for the amino acid sequence represented by formula I, with preference given to the nucleotide sequence represented by the following formula II:

30

5' -CGAAATTCAACCTCCCGATAGGGCTGGGCGTGACCAAAAATATACTGGGTTCTGTTCCTTTTCTG
ATCATTCTTACAAGTTATACTCTTATTTGGAAGGCCCTAAAGAAGGCTTATGAAATTCAGAAGAACAACCCA
AGAAATGATGATATTTTTAGGATAATTATGGCAATTGTGCTTTTCTTTTCTTTTCTGGATTCCCCACCAA
ATATTCACTTTTCTGGATGTATTGATTCAACAGGGCATCATACGTGACTGTAGAATTGCAGATATTGTGGAC
35 ACGGCCATGCCCATCACCATTGGATAGCTTATTTAACAATTGCCTGAATCCTCTGTTTTATGGCTTTCTG
GGAAAAAATTTAAAAAGATATT-3'

(SEQ ID No. 2)

5 Recombinant DNA (A) is preferably the DNA represented by the nucleotide sequence shown in Fig. 1 (SEQ ID No. 5). Any nucleotide sequence can serve to code for the human Ang II type 1 receptor represented by the amino acid sequence shown in Fig. 2, with preference given to the nucleotide sequence represented by the following formula (which is same as nucleotides at the 240-1316 positions in the nucleotide sequence shown in Fig. 1):

10

5' -ATGATCCTCAACTCTTCTACTGAAGATGGTATTAAGAAGATCCAAGATGATTGTCCCAAAGCTGGAA
GGCATAATTACATATTTGTCATGATTCTACTTTATACAGTATCATCTTTGTGGTGGGAATATTTGGAAA
CAGCTTGGTGGTGATAGTCATTTACTTTTATATGAAGCTGAAGACTGTGGCCAGTGTTCCTTTTGAAT
15 TTAGCACTGGCTGACTTATGCTTTTACTGACTTTGCCACTATGGGCTGTCTACACAGCTATGGAATACC
GCTGGCCCTTTGGCAATTACCTATGTAAGATTGCTTCAGCCAGCGTCAGTTTCAACCTGTACGCTAGTGT
GTTCTACTCACGTGTCTCAGCATTGATCGATACCTGGCTATTGTTCAACCAATGAAGTCCCGCCTTCGA
CGCACAATGCTTGTAGCCAAAGTCACCTGCATCATCATTGGCTGCTGGCAGGCTTGGCCAGTTGCCAG
20 CTATAATCCATCGAAATGTATTTTTCATTGAGAACACCAATATTACAGTTTGTGCCTTCCATTATGAGTC
CCGAAATTCAACCCTCCCGATAGGGCTGGGCCTGACCAAAAATATACTGGGTTCTGTTTCCCTTTTCTG
ATCATTCTTACAAGTTTACTCTTATTTGGAAGGCCCTAAAGAAGGCTTATGAAATTCAGAAGAACAACC
25 CAAGAAATGATGATATTTTATAGGATAATTATGGCAATTGTGCTTTTCTTTTCTTTTCTGGATTCCCCA
CCAAATATTCATTTTCTGGATGTATTGATTCAACAGGGCATCATACGTGACTGTAGAATTGCAGATATT
GTGGACACGGCCATGCCATCACCATTGGATAGCTTATTTTAACAATTGCCTGAATCCTCTGTTTTATG
GCTTTCIGGGAAAAAATTTAAAAAGATATTCTCCAGCTTCTGAAATATATTTCCCCAAAGGCCAAATC
30 CCACTCAAACCTTTCAACAAAAATGAGCACGCTTTCCTACCGCCCCTCAGATAATGTAAGCTCATCCACC
AAGAAGCCTGCACCATGTTTTGAGGTTGAG-3'

35

A vector harboring recombinant DNA containing a gene which codes for the human Ang II type 1 (hAT₁) receptor of the present invention can, for example, be produced by:

- (a) separating RNA which codes for the hAT₁ receptor,
- 5 (b) synthesizing single-stranded complementary DNA (cDNA) from said RNA and then double-stranded DNA,
- (c) incorporating said double-stranded DNA to an appropriate plasmid,
- (d) transforming an appropriate host with the resulting recombinant plasmid,
- 10 (e) culturing the resulting transformant and then isolating the plasmid containing the desired DNA therefrom by an appropriate method (e.g., colony hybridization using a DNA probe),
- (f) cleaving out the desired cloned DNA from the plasmid, and
- (g) ligating said cloned DNA to the downstream of an appropriate promoter
- 15 in a vehicle.

The cDNA can also be produced by chemical synthesis.

RNA which codes for a human Ang II type 1 receptor can be obtained from various human organs and cells. Methods of preparing RNA from these
20 materials include the guanidine thiocyanate method [J.M. Chirgwin et al., Biochemistry, 18, 5294 (1979)].

After adding an oligo-dT primer or random oligonucleotide to the thus-obtained RNA, reverse transcriptase may be added to synthesize cDNA [H. Okayama et al., Molecular and Cellular Biology, 2, 161 (1982) and 3, 280
25 (1983)]. To this cDNA preparation, sense primer and antisense primer (see Examples below) may be added to amplify a well-conserved region, based on the reported sequences for the Ang II type 1 receptor from bovine adrenal zona glomerulosa cells [K. Sasaki et al., Nature, 351, 230 (1991)] and for the Ang II type 1 receptor from rat aortic smooth muscle cells [T.J. Murphy et al.,
30 Nature, 351, 233 (1991)]. Then, a polymerase chain reaction (PCR) may be carried out using a commercially available kit (e.g., a kit produced by Cetus/Perkin-Elmer). The amplified cDNA can be separated by a known method such as agarose electrophoresis and then recovered from the gel. The nucleotide sequence of this cDNA can be determined by, for example, the
35 "dideoxy" chain termination method [T. Messing et al., Nucleic Acids Research, 9, 309 (1981)].

The plasmid having the cloned cDNA may be used as such or after being cleaved with appropriate restriction enzyme and inserted to another vector (e.g., plasmid) as necessary.

5 Examples of the plasmid for cDNA insertion include plasmids derived from *Escherichia coli* such as pBR322 [Gene, 2, 95 (1977)], pBR325 [Gene, 4, 121 (1978)], pUC12 [Gene, 19, 259 (1982)], pUC13 [Gene, 19, 259 (1982)], pUC118 and pUC119 [Methods in Enzymology, 153, 3-11 (1987)] and those derived from *Bacillus subtilis* such as pUB110 [Biochemical and Biophysical
10 Research Communications, 112, 678 (1983)], but any other can be used for this purpose, as long as it is replicable and retainable in the host.

 Examples of the method of insertion to the plasmid include that described by T. Maniatis et al. in Molecular Cloning, Cold Spring Harbor Laboratory, page 239 (1982).

15 The plasmid incorporating said cDNA may be a plasmid obtained using a cDNA library (with *Escherichia coli* x1776) host prepared by inserting a cDNA synthesized from human normal diploid cell mRNA to the pCD vector [see Okayama et al., Molecular Cell Biology, 3, 280 (1983)], which cDNA library was given from Dr. Okayama at the Research Institute for Microbial
20 Diseases, Osaka University.

 The plasmid thus obtained is introduced to an appropriate host such as a bacterium of the genus *Escherichia* or *Bacillus*.

25 Example bacteria of the genus *Escherichia* include *Escherichia coli* K12DH1 [Proceedings of the National Academy of Science, USA, 60, 160 (1968)], M103 [Nucleic Acids Research, 9, 309 (1981)], JM109 [Methods in Enzymology, 153, 3-11 (1987)], JA221 [Journal of Molecular Biology, 120, 517 (1978)], HB101 [Journal of Molecular Biology, 41, 459 (1969)], C600 [Genetics, 39, 440 (1954)] and MC1061/P3 [Nature, 329, 840 (1987)].

30 Example bacteria of the genus *Bacillus* include *Bacillus subtilis* MI114 [Gene, 24, 255 (1983)] and 207-21 [Journal of Biochemistry, 95, 87 (1984)].

 Methods of transformation include the calcium chloride method and calcium chloride/rubidium chloride method described by T. Maniatis in Molecular Cloning, Cold Spring Harbor Laboratory, page 249 (1982).

35

From the transformants thus obtained, the desired clone is selected using a known method, such as colony hybridization [Gene, 10, 63 (1980)] or DNA nucleotide sequencing [Proceedings of the National Academy of Science, USA, 74, 560 (1977); Nucleic Acids Research, 9, 309 (1981)].

5 A microorganism carrying a vector containing a nucleotide sequence which codes for the cloned hAT₁ receptor is thus obtained.

Next, the plasmid is isolated from the microorganism. Methods of such isolation include the alkali method [H.C. Birnboim et al., Nucleic Acids Research, 1, 1513 (1979)].

10 The above vector containing a gene which codes for the cloned hAT₁ receptor can be used as such or after being cleaved out with restriction enzyme as necessary.

The cloned gene is joined to the downstream of the promoter, in a vehicle (vector) suitable for its expression, to yield an expression vector.

15 Example vectors include the above-mentioned plasmids derived from *Escherichia coli* (e.g., pBR322, pBR325, pUC12, pUC13, pUC118, pUC119), plasmids derived from *Bacillus subtilis* (e.g., pUB110, pTP5, pC194), yeast-derived plasmids (e.g., pSH19, pSH15), bacteriophages such as λ phage, 20 animal viruses such as retrovirus and vaccinia virus and plasmids for animal cell expression (e.g., pcDNA I).

The gene may have ATG (nucleotide sequence which codes for an appropriate signal peptide as desired) as a translational initiation codon at its 5'-terminal and TAA, TGA or TAG (preferably TGA) as a translational 25 termination codon at its 3'-terminal. To express the gene, a promoter is ligated to the upstream thereof. Any promoter can be used for the present invention, as long as it is appropriate for the host used to express the gene. Examples of preferred promoters are given below.

30 Preferred promoters include the T7 promoter, trp promoter, lac promoter, rec A promoter, λ P_L promoter and lpp promoter, when the transformation host belongs to the genus *Escherichia*; the SPO1 promoter, SPO2 promoter and pen P promoter when the host belongs to *Bacillus*; and the PHO5 promoter, PGK promoter, GAPDH promoter and ADH promoter 35 when the host is a yeast. Preference is given to the case in which a bacterium of the genus *Escherichia* is used as host in combination with the trp promoter

or T7 promoter. When the host is an animal cell, preferable promoters include the SV40-derived promoter, retrovirus promoter and human cytomegalovirus promoter, with preference given to the SV40-derived promoter.

5

The thus-constructed expression vector, containing a gene encoding an hAT₁ receptor, is used to produce a transformant. Examples of the host include bacteria of the genus *Escherichia*, bacteria of the genus *Bacillus*, yeasts and animal cells, with preference given to animal cells. Examples of
10 the bacteria of the genus *Escherichia* and of the genus *Bacillus* include the same as specified above. Especially, the host is preferably an animal cell, when the transformant cells are used for screening an agonist or an antagonist of the hAT₁ receptor as described below.

Examples of the yeasts include *Saccharomyces cerevisiae* AH22R-,
15 NA87-11A and DKD-5D. Example animal cells include simian cells COS-7, Vero, Chinese hamster ovary (CHO) cells, mouse L cells, mouse myeloma Sp2/O cells and human FL cells.

The bacteria of the genus *Escherichia* can be transformed in accordance with the method described in the Proceedings of the National Academy of Science, USA, 69, 2110 (1972), Gene, 17, 107 (1982), for instance. Bacteria of
20 the genus *Bacillus* can be transformed in accordance with the method described in Molecular and General Genetics, 168, 111 (1979), for instance. Yeasts can be transformed in accordance with the method described in the Proceedings of the National Academy of Science, USA, 75, 1929 (1978), for
25 instance. Animal cells can be transformed in accordance with the method described in Virology, 52, 456 (1973), for instance.

A transformant resulting from transformation with a vector harboring the cDNA of hAT₁ receptor is thus obtained.

30

For cultivating a transformant whose host is a bacterium of the genus *Escherichia* or *Bacillus*, it is appropriate to use a liquid medium supplemented with carbon sources, nitrogen sources, minerals and other substances necessary for transformant growth. Example carbon sources include glucose, dextrin, soluble starch and sucrose. Example nitrogen
35 sources include organic or inorganic substances such as ammonium salts, nitrates, corn steep liquor, peptone, casein, meat extract, soybean cake and

potato extract. Example minerals include calcium chloride, sodium dihydrogen phosphate and magnesium chloride. Yeast extract, vitamins, growth factors and other additives may be added. The pH of the medium is preferably about 6 to 8.

5

Examples of media preferably used to cultivate the genus *Escherichia* include M9 medium containing glucose and Casamino acid [Miller, Journal of Experiments in Molecular Genetics, 431-433, Cold Spring Harbor Laboratory, New York (1972)]. To increase promoter efficiency as necessary, a chemical agent such as 3 β -indolyl acrylic acid may be added.

10

When the host is a bacterium of the genus *Escherichia*, cultivation is normally carried out at about 15 to 43°C for about 3 to 24 hours, with aeration and/or stirring as necessary.

15

When the host is a bacterium of the genus *Bacillus*, cultivation is normally carried out at about 30 to 40°C for about 6 to 24 hours, with aeration and/or stirring as necessary.

20

Examples of media for cultivating a transformant whose host is a yeast include Burkholder's minimal medium [Bostian, K.L. et al., Proceedings of the National Academy of Science, USA, 77, 4505 (1980)]. The preferable pH of the medium is pH of about 5 to 8. Cultivation is normally carried out at about 20 to 35°C for about 24 to 72 hours, with aeration and/or stirring as necessary.

25

Examples of media for cultivating a transformant whose host is an animal cell include MEM medium [Science, 122, 501 (1952)], DMEM medium [Virology, 8, 396 (1959)], RPMI 1640 medium [Journal of the American Medical Association, 199, 519 (1967)], 199 medium [Proceedings of the Society for the Biological Medicine, 73, 1 (1950)] and ASF104 (produced by Ajinomoto). These media may be supplemented with about 5 to 20% fetal bovine serum. The pH is preferably about 6 to 8. Cultivation is normally carried out at about 30 to 40°C for about 15 to 60 hours, with aeration and/or stirring as necessary.

30

Separation and purification of the hAT₁ receptor of the present invention from the culture described above can, for example, be achieved as follows:

35

In extracting the hAT₁ receptor of the present invention from cultured cells, the cells are collected by a known method after cultivation and suspended in a buffer containing a protein denaturant, such as guanidine hydrochloride, to elute the desired hAT₁ receptor from the cells. In another method, the cells are disrupted by ultrasonication, lysozyme treatment and/or freeze-thawing, after which they are centrifuged to separate the receptor of the invention. The method using a combination of lysozyme treatment and ultrasonication is preferred.

For purifying the receptor of the present invention from the supernatant, known methods of separation and purification can be used in combination as appropriate. Such known methods of separation and purification include those based on solubility differences such as salting-out and solvent precipitation, those based mainly on molecular weight differences such as dialysis, ultrafiltration, gel filtration and SDS-polyacrylamide gel electrophoresis, those based on charge differences such as ion exchange chromatography, those based on specific affinity such as affinity chromatography, those based on hydrophobicity differences such as reverse-phase high performance liquid chromatography, and those based on isoelectric point differences such as isoelectric focusing.

The thus-obtained hAT₁ receptor of the present invention may be prepared as a dry powder by dialysis, lyophilization and other treatments. It is appropriate to add serum albumin etc. as a carrier in storing the hAT₁ receptor, since its adsorption to the container is prevented.

The hAT₁ receptor of the present invention is thus obtained, in a substantially pure form. The substantially pure protein of the present invention has a protein content of not less than 95% (w/w), preferably not less than 98% (w/w).

The hAT₁ receptor thus obtained, the cells containing, specifically expressing, the receptor, cell membrane preparations therefrom, or the like can be used for screening of substances exhibiting antagonist or agonist activity against the hAT₁ receptor, in which the amount of ligand bound to the hAT₁ receptor is determined in a ligand binding test, for instance. Especially, it is suitable for searching an anti-Ang II substance which inhibits the binding of Ang II to the receptor in the presence of the hAT₁ receptor of the present invention and Ang II as the ligand. Since thus obtained animal

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